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# Microarray Analysis of Gene Expression Essential to Energetic Efficiency in a Porcine Model of Obesity

**A.S. Leaflet R2041**

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## Summary and Implications

To better understand the molecular mechanisms underlying energetic efficiency and obesity development in mammals, genes differentially expressed in a long-term, porcine model of obesity development were evaluated. The model consisted of eighty pigs from a single genetic strain and rearing environment receiving a caloric intake equivalent to 1.8 times their daily body maintenance from a nutrient mix representative of an American diet for 144 days. Pigs (8/group) with the highest and lowest energetic efficiency (EE) were identified and differential gene expression of over 10,000 gene transcripts in adipose, muscle and liver tissues was evaluated via a porcine specific microarray. Dietary ME intakes and body weight gains were similar between the two EE groups but high EE animals accrued 52% more energy and 76 % more body fat tissue. Microarray analysis revealed marked differences ( $p < 0.05$ ;  $> 1.5$  fold difference) in expression of over 100, 200, and 150 genes in adipose, muscle and liver tissues, respectively.

Expression of genes involved in fatty acid metabolism were expressed at significantly higher levels in the low EE group, whereas expression of some genes involved in ATP synthesis, lipogenesis and cholesterol synthesis, were expressed at significantly higher levels in the high EE group. Interestingly, genes implicated in defense and stress responses, such as heat shock proteins and some cytokines, were also expressed at higher levels in the high EE group. Additionally, a number of previously unappreciated regulatory molecules were identified that may be influencing energetic efficiency in mammals independent of energy intake. These results aid in the identification of novel genes and molecules involved in modulating energetic efficiency and the development of the obesity phenotype in mammals independent of energy intake.

## Introduction

Energetic efficiency is important in animal production and in human health. Obesity or excess body fat accretion occurs when energy intake exceeds energy expenditure over an extended period of time. Recent evidence indicates that substantial differences exist among individuals in their metabolic energy expenditure independent of their energy intake. These differences in metabolic efficiency are assumed to be due in part to differences in tissue specific gene expression.

The objective of this project is to identify genes that are differentially expressed in relation to efficiency of energy accretion/use and to identify possible targets for bioactive molecules for the purpose of modifying metabolic efficiency and physiological health of mammals.

## Methods and Materials

Pigs from a porcine model of obesity development were evaluated. Specifically, eighty pigs (initial body weight of 154 kg) from a single genetic strain and rearing environment were individually provided daily caloric intakes equivalent to 1.8 times body maintenance needs for 144 days from a dietary nutrient mix representative of an American diet (34% of calories from fat). The model was developed to simulate the amount of additional body fat (25-28 kg) accrued in adolescents and in adults for obesity development to occur.

Following 144 days on the dietary regimen, pigs were sacrificed and samples taken from adipose, muscle and liver for RNA isolation. Body tissue compositions were also determined via Dual Energy X-ray Absorptionmetry (DEXA). The sample population of animals with the lowest and highest energetic efficiency (energy retained in body tissue/ ME intake consumed above the animals body maintenance needs) were identified. Tissues from these selected animals (8/EE group) were then subjected to RNA isolation for subsequent gene expression analysis.

Total RNA was isolated from liver and muscle samples using Trizol (Invitrogen Co., Carlsbad CA) as recommended by the manufacturer. Because concentrations of RNA in adipose tissue are very low in comparison to liver or muscle, adipose RNA was isolated using the method reported by Chomczynski and Sacchi (1987) which allows for much larger quantities of starting material. Quantity and quality of isolated RNA samples were analyzed by spectrophotometry at 260 and 280nm. Quality was further assayed by RNA separation using agarose gel electrophoresis and visualization utilizing RNA fluorescence by ethidium bromide and UV light.

In duplicate, reverse transcriptase was used to convert liver, muscle and adipose total RNA (25, 40 and 40ug, respectively) into cDNA probes with the incorporation of an aminoallyl modified dUTP. In separate reactions, probes were conjugated to either Cy3 or Cy5 fluorescent markers (by binding to the aminoallyl modified dUTP). By using the labeled cDNA to probe a porcine oligo microarray (developed by Qiagen Inc.) in a competitive manner for 18-20 hours, it is possible to determine relative amounts of expression levels between EE groups for all genes represented on the microarray (over 10,000 unique genes, several hundred positive and

negative control genes and several hundred blank controls).

Digital pictures were taken of the microarrays using the available GSI Lumonics ScanArray 5000 (Perkin Elmer) and ScanArray 3.1 software (Packard Biochips Technologies) at Iowa State University's Center for Plant Genomics Microarray Facility to obtain fluorescence images for both Cy3 and Cy5 binding to each spot on the array. These fluorescence images were analyzed using ImageJ 6.0 software (BioDiscovery Inc.) to obtain two intensity values for each of the 14,688 spots on the microarray. These intensity values (one for Cy3 and one for Cy5) were then used as raw data to determine the levels of gene expression for each pig within the study.

Within each tissue, intensity values from the modified loop design (see Figure 1) were normalized using the LOESS method. Normalized values were then subjected to a SAS mixed model analysis that took into account the fixed factors of efficiency of energy utilization and dye bias. Random effects of the model were loop, microarray nested within loop, and individual pig nested within loop nested within energy utilization. The Satterthwaite method (Satterthwaite, 1946) was used to determine denominator degrees of freedom for all F-tests associated with each mixed linear model analysis. Estimates of EE effects were obtained by contrasting appropriate means. These estimates were converted to the fold difference scale by exponentiation of the estimated log-scale difference in means. Differences in gene expression between EE levels were deemed significant at  $p < 0.05$  with an absolute expression difference of 1.5 fold

### Results and Discussion

Body weights, energy intake, energy utilization and tissue accretion of pigs in the high and low EE groups are reported in Table 1. As designed, the ME intake (above body maintenance needs) of each animal was the same in both groups. However, pigs in the high EE group accrued 52% more energy and 76 % more fat tissue in their bodies than the low EE animals. Specifically, animals in the high EE group accrued 0.844 units of body energy for each Mcal of ME consumed above maintenance compared with only 0.444 units in the low EE groups. These data demonstrate that substantial differences in energy expenditure and resultant fat tissue accretion occur between individuals independent of dietary energy intake.

Using p-value and fold difference as our criteria to determine gene expression differences between EE outcomes, over 100 differentially expressed genes in adipose and over 200 and 150 in glycolytic muscle and liver tissue, respectively, were observed.

Several liver specific genes that had higher expression in the high EE pigs also have interesting functional roles. Two mitochondrial protein genes, mitochondrial ribosomal protein L19 (FD>2.64) and mitochondrial ribosomal protein L42 (FD>1.69) are

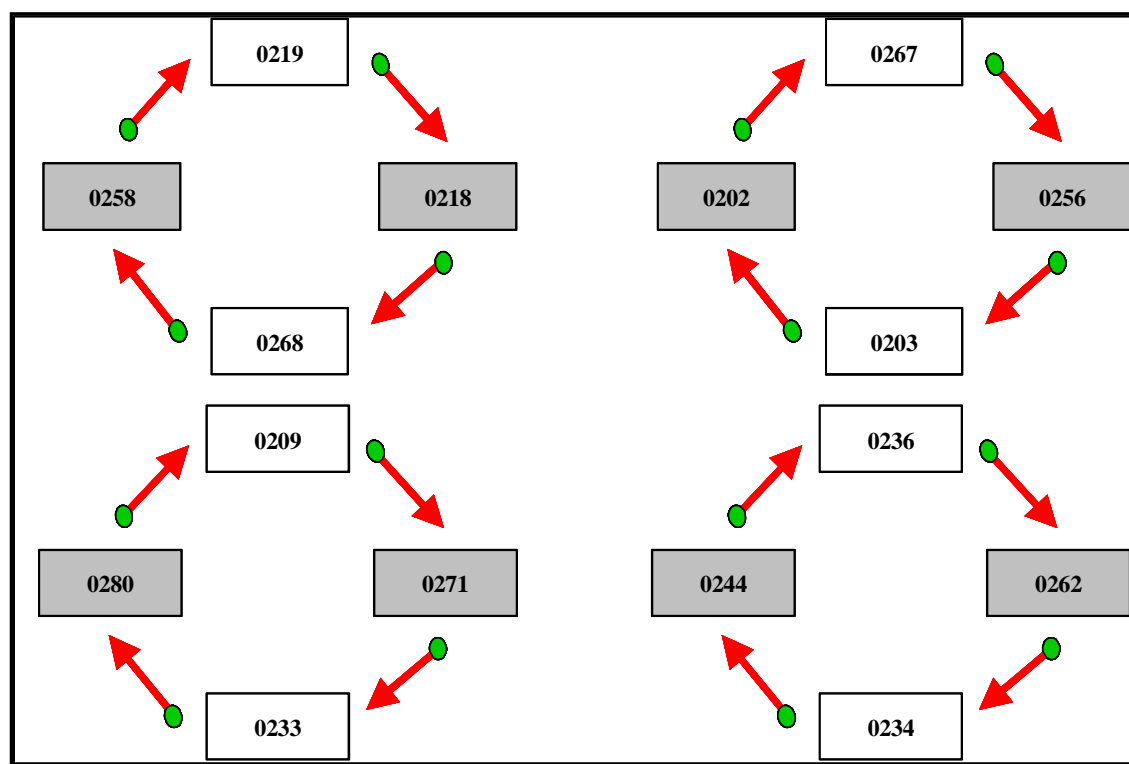
expressed at higher levels in high EE pigs. These genes are encoded by the nucleus but function in protein synthesis in the mitochondria. GAPDH, historically a control for normalizing gene expression data but is currently recognized as malleable by many stimuli, appears in high EE pigs at levels twice that of low EE pigs.

As with liver, in glycolytic muscle there were several genes whose expression differences appeared interesting. One group of genes whose products have been shown to have roles in synthesis, regulation or stability of the protein actin, were each expressed at higher levels in the high EE pigs. These genes included TUBB (FD>1.58), endozepine (FD>4.0), nesprin-2 (FD>3.17) and actin binding LIM protein 1 (FD>1.74). A second group of genes having to do with cellular transport also came up as significant ( $p < 0.05$ ) but in the opposite direction (that is higher in the low EE pigs) as the genes involved with actin. These genes included syntaxin (FD<1.75), COP9 (FD<1.72) and acyl CoA synthetase.

With just over 100 significant differences in gene expression ( $p < 0.05$ ) between EE levels, adipose had the least amount of differential expression of three tissues analyzed. However, differential expression of several genes directly related to fat metabolism and energy utilization were observed. These included higher expression of fatty acid synthase (FD<2.39), acyl CoA carboxylase alpha (FD<2.08), acyl CoA carboxylase (FD<2.02), and ELOVL 5 and 6 (both FD<1.5) in the low EE pigs. Since the American diet is very high in available fat calories (34%), it is possible that high EE pigs would directly incorporate more dietary fat into body tissues and subsequently express these genes at lower levels than low EE pigs.

Interestingly Integrin alpha-7 (cellular adhesion molecule) was shown to be expressed significantly lower in high EE pigs in both liver and muscle (FD<1.67 and FD<1.77 respectively). The only other gene expression event that was not tissue specific was acyl coenzyme A synthetase. This gene product has a role in fat mobilization and was shown to be expressed significantly lower in high EE pigs in both muscle and fat tissues (FD<2.14 and FD<1.77 respectively).

At this point in the study we have only just begun to theorize how these differences in gene expression may effect energy utilization and the resultant changes in body tissue content and physiological health. The next component of this study will be to validate selected gene expression events uncovered by microarray analysis. To do this our group will employ real time PCR. This technique, which is far more sensitive the microarray analysis, will yield a more detailed picture of energy utilization gene expression. It is our ultimate goal that this set of experiments will yield a unique perspective on opportunities to optimize energetic efficiency and health of mammals.



**Figure 1:** RNA from high energetic efficiency pigs (grey boxes) was compared with RNA from low energetic efficiency pigs (white boxes) in a flip-flour manner. The arrows between each box represent a microarray preformed using RNA from each box (arrow head is labeled Cy5 while the arrow tail is labeled Cy3) so that all samples were labeled with both Cy3 and Cy5 at one point in the loop.

Criteria		Low	High	Difference
No of pigs		8	8	
Initial Weight (kg)		155.8	154.1	-1.7
Final Weight (kg)		245.0	244.0	-1.0
Weight Gain (kg)		89.2	89.9	0.7
Carcass Weight Gain (kg)		75.6	76.9	1.3
<b>Body Tissue Gain (kg)</b>				
Fat		25.8	45.4	19.6
Lean		47.7	31.2	-16.5
Total		74.2	77.3	3.1
<b>Body Gain Composition, % of DEXA Estimated Body Weight Gain</b>				
Fat		34.7	58.7	24.0
Lean		64.3	40.4	-23.9
<b>Subcutaneous Backfat (mm)</b>				
Last rib		32.0	43.0	11.0
<b>Energetic Efficiency [tissue energy gain/ME intake above maintenance (MEam)]</b>				
MEam Intake (Mcal)		532.0	533.0	1.0
Energy Gain (Mcal)		292.0	444.0	152.0
Energy Gain/MEam		0.544	0.842	0.340

**Table 1: Characterization of Low and High Energetic Efficiency (EE) Pigs.**

Values reported in this table represent a subset of pigs (8/EE) with the highest and lowest energetic efficiency [energy accrued in body tissues/ME consumed above maintenance (MEam)]. Energy accretion was calculated from dual-energy x-ray absorptiometry determined body tissue contents and assumed energy content of fatty and lean (fat free) tissues. Tissue content of pigs at initiation of the study was estimated from the relationship of tissue contents and body weights determined in a subsample of pigs killed at the initiation of the study. The ME content of the diet was determined from the NRC (1998) ME values for each dietary ingredient. Pig's ME intake above maintenance was determined from the pig's actual ME intake minus the pig's body maintenance ( $110 \text{ kcal ME/body weight, kg}^{0.75}/\text{day}$ ) needs.